The Minimal Set of Genetic Alterations Required for Conversion of Primary Human Fibroblasts to Cancer Cells in the Subrenal Capsule Assay[1]

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Abstract

Based on previous studies, a minimal set of genetic alterations that is required to convert normal human fibroblasts into cancer cells has been defined. Essential roles for telomere maintenance and alterations in phosphatase 2A activity were inferred from experiments in which tumorigenicity was tested by injecting cells under the skin of immunodeficient mice. However, in the present experiments, the combination of SV40 large T antigen and activated Ras, without hTERT or SV40 small t antigen, was sufficient to convert nine different primary human fibroblast cell strains to a fully malignant state. The malignant behavior of the cells was demonstrated by growth of the cells into invasive tumors when the cells were injected beneath the kidney capsule of immunodeficient mice. Lung metastases and circulating tumor cells were also detected. These tumors were not immortal; cells entered crisis, from which they could be rescued by expression of hTERT. However, the same cell populations were not tumorigenic when they were injected under the skin. In this site, tumorigenicity required the expression of hTERT and SV40 small t antigen as well as SV40 large T antigen and Ras. The cellular pathways targeted by SV40 large T antigen (p53 and pRb) and those targeted by activated Ras represent a minimal set of genetic alterations required for the conversion of normal human fibroblasts into cancer cells.

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Introduction

To define genetic alterations needed to convert a normal human cell into a cancer cell, it is necessary to have a reliable method for assessing the tumorigenic potential of genetically modified cells. Obviously, in the case of human cells, tumorigenic potential must be tested by growth of the cells as a xenograft in immunodeficient mice, rather than by growth in a syngeneic host as is possible for experimental animals. Beginning with the introduction of the nude (athymic) mouse into cancer research, it became routine to assay tumorigenicity by injection of cells subcutaneously or intramuscularly. For human cells, the definition of an oncogene became firmly linked to the assay of subcutaneous growth in immunodeficient animals.

Using subcutaneous growth to define the genes needed to make a cancer cell, it was found that the combination of four genetic elements—SV40 large T antigen (LT), SV40 small t antigen (ST), oncogenic *Ras*, and human telomerase reverse transcriptase subunit (*hTERT*)—represented a minimal set of genes required for the neoplastic transformation of primary human fibroblasts and several other human cell types [1–3]. These experiments yield the conclusion that the cellular pathways or processes that are perturbed by the introduction of these genes define a minimal set of cellular/molecular alterations needed to convert normal human fibroblasts into cancer cells. These alterations involve the maintenance of telomeres by telomerase, inactivation of the retinoblastoma (pRB) and p53 tumor-suppressor pathways (by SV40 LT), acquisition of a constitutive mitogenic signal provided by oncogenic Ras, and perturbation of protein phosphatase 2A by SV40 ST [2,3].

Although injection of cells beneath the skin of immunodeficient mice has been the predominant method of assessing the tumorigenicity of human cells, there is no reason to assume that this site is optimal for tumor formation by implanted cells. Other sites in the body may provide a better microenvironment for tumor growth. In particular, the kidney has long been known to support the growth of primary human tumors [4]. Following the success of the growth of solid tumor fragments in the kidney, the technique was adapted for cultured cells by embedding them in a fibrin clot before they were inserted under

the kidney capsule [5]. More recently, the subrenal capsule space has been used for tissue recombination and reconstruction, as applied to breast and prostate tissues [6,7]. However, even before these techniques were introduced, it had already been demonstrated that cancer cells will grow into tumors when injected as cell suspensions beneath the capsule of the kidney [8,9]. Studies in this laboratory on adrenocortical cells showed that injection of cells under the capsule of the kidney was ideal for cell survival, permitting functional vascularized tissue formation from normal primary cells [10–12].

Using the subrenal capsule assay for assessing tumorigenicity, we found that bovine and human adrenocortical cells could form invasive and metastatic tumors with just two of the four genes, *SV40 LT* and *Ras* [13]. It was necessary to prove that the tumors were truly telomerase-negative and lacked a telomere maintenance mechanism. Because bovine cells have longer telomeres than human cells, tumors that were formed from bovine cells were much larger than those formed from human adrenocortical cells. This enabled two experiments that were not feasible with the human adrenocortical tumors; first, it was possible to flow sort cells from the primary tumor and obtain sufficient cells for an assay of telomerase activity, and, second, it was possible to serially transplant the tumors to secondary, and sometimes tertiary, host animals. These experiments proved that the cells truly lack a telomere maintenance mechanism, thus eliminating telomerase as an essential component of the cellular pathways and processes that are needed for the tumorigenic conversion of this cell type.

However, when we used subcutaneous growth, rather than growth in the subrenal capsule assay, as the test of tumorigenicity in genetically modified bovine adrenocortical cells, we found that hTERT was required together with SV40 LT and Ras. This suggested, consistent with other observations [14], that hTERT has effects on cellular behavior that are not linked to its effects on telomere maintenance. We also found that subcutaneous growth of genetically modified bovine adrenocortical cells did not require SV40 ST [13].

In the present experiments, we set out to clarify whether these findings—that hTERT is needed for subcutaneous tumor growth, but only two genes, *SV40 LT* and *Ras*, are required for growth in the subrenal capsule assay—are true for a more widely studied human cell type, the primary human fibroblast. Prior studies have suggested that some tumorigenic growth of genetically modified primary human fibroblasts is possible without the introduction of telomerase [15,16]. However, in these experiments, it was not shown that the tumors truly lacked a telomere maintenance mechanism, or that the progressive loss of malignant properties could be reversed by the introduction of hTERT, as we did for adrenocortical cells [13]. To our knowledge, the ability of genetically modified human fibroblasts to grow into tumors in the subrenal capsule site has not previously been studied. Here we compared the properties of primary human fibroblasts transduced with combinations of SV40 LT, ST, Ras, and hTERT in subrenal capsule transplantation and conventional subcutaneous injection.

Materials and Methods

Growth of Human Fibroblasts and Retroviral Transduction

Primary human fibroblasts were obtained as follows: HCA2 (MJ-90) and HCA3 (BJ) were derived from the laboratory of Dr. Olivia Pereira-Smith (UTHSCSA, Houston, TX); LF1 was a generous gift of Dr. John Sedivy (Brown University); CRL-2088 (CCD-1072sk), CRL-2091 (CCD-1070sk), CRL-2094 (CCD-1077sk), CRL-2097 (CCD-1079sk), CRL-2429 (CCD-1112sk), and CRL-2703 (CCD-1137sk) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The retroviruses used are of the LX type [17] and have been described previously [13]. They were constructed from pLEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) by replacing the *neo* gene. SV40 large T antigen (*SV40 LT*) was derived from an intronless cDNA. A second version of this retrovirus was constructed using the intron-containing early region SV40 DNA. The oncogenic *Ras* used is Ha-Ras^{G12V}. Retroviral plasmid pBabe-puro-hTERT was generously donated by J. Campisi. The Phoenix cell line (amphotropic) was used for the production of retroviral particles [18]. Phoenix cells were transfected with retroviral plasmids by the calcium phosphate method. After 48 hours, the supernatant medium was filtered through a 0.45-μm filter and added to the target cells. Infection was allowed to proceed for 48 hours. Because the retroviral constructs used encode GFP, the rate of infection of the culture was monitored by fluorescence microscopy.

Pure populations of transduced cells were prepared by flow cytometry. More than 98% of the cells were fluorescent after sorting. Sorted cells were expanded in culture for transplantation or for biochemical studies. No drug selection was used to introduce SV40 LT, ST, or Ras; but when pBabe-hTERT was used, cells were selected with 1 μg/ml puromycin.

Anchorage-independent growth was assayed by growth in soft agarose [19]. Cells were plated in medium containing 0.4% low melting agarose over an underlay of 0.8% agarose. After the agarose solidified, medium was added to the top surface and replaced every 2 days.

Biochemical Characterization of Retrovirally Transduced Cells

Western blot analysis was performed using standard techniques. Antibodies used were: SV40 LT, mouse monoclonal PAb419 (EMD Biosciences, La Jolla, CA); and Ras, rat monoclonal Y13-259 (EMD Biosciences). Telomerase activity in detergent extracts of cells was assessed by the telomerase repeat amplification protocol (TRAP) assay as previously described [13]. Telomere restriction fragment (TRF) analysis was performed as previously described [11] with the exception that DNA fragments were separated by pulse-field gel electrophoresis.

Transplantation of Cells in RAG2$^{-/-}$,γc$^{-/-}$ Mice

RAG2$^{-/-}$,γc$^{-/-}$ mice were purchased from Taconic (Germantown, NY). Animals (both males and females) at an age greater than 6 weeks (~ 25 g body weight) were used in these experiments. Procedures were approved by the institutional

animal care committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. We transplanted 2×10^6 fibroblasts beneath the kidney capsule as previously described for adrenocortical cells [10–12]. Subcutaneous cell implantation was performed using a 29-gauge needle. Potential leakage of cells from the injection site was monitored by illumination with a 470-nm light source (Lightools Research, Encinitas, CA).

Animals were sacrificed at various times following transplantation, as described in the Results section. Tumors were visualized by fluorescence using a 470-nm light source in conjunction with a low level of white light. This combination allowed the direct visualization of the fluorescent tumor cells together with the nonfluorescent organs.

Histologic and Immunohistochemical Analysis

The fixation, paraffin embedding, and histologic examination of tissue formed from transplanted cells were carried out using standard techniques. SV40 LT was detected with monoclonal antibody PAb416 (EMD Biosciences). Additionally, tissues were fixed using the AMeX method [20]. Thick (30–50 μm) sections were prepared from AMeX-fixed, paraffin-embedded tissues and were used for immunocytochemistry without antigen retrieval. Antibodies against 53BP1 and γ-H2AX were obtained from Upstate Biotechnology (Lake Placid, NY). Bound antibodies were visualized with biotinylated secondary antibodies and streptavidin–fluorochrome conjugates (Vector Laboratories, Burlingame, CA). Photography was performed using a Zeiss Axiovert fluorescence microscope.

Results

Normal Human Fibroblasts Can Be Transformed to a Fully Malignant State by SV40 LT and Ras^G12V

Nine different strains of normal human fibroblasts were transduced with a combination of retroviruses encoding SV40 LT and Ras^G12V. Fibroblasts were used at the earliest passage level available for each strain. At the time of retroviral infection, all the cell populations used had a labeling index of >90% (based on a 72-hour incubation in bromodeoxyuridine) and a remaining division potential before senescence of at least 20 population doublings (PD) (data from our own observations and data provided by the ATCC). One of the retroviral constructs (Ras^G12V) also encoded GFP. Following selection by fluorescence-activated cell sorting, cells were injected beneath the kidney capsule of immunodeficient mice. Animals were sacrificed at 30 to 70 days following cell transplantation. All SV40 LT/Ras^G12V–transduced fibroblast strains produced tumors that grew beneath the kidney capsule (Figure 1; Table 1). Histologic sections of all tumors showed invasion of the oncogene-expressing cells into the kidney. Additionally, separate tumor masses within the peritoneal cavity were detected in many animals, and in a smaller number, metastases to the lungs were observed by fluorescence and confirmed by histology (Figure 1). Circulating tumor cells were observed in the spleen by immuno-

cytochemistry. Although death was not a planned endpoint of the experiments, some animals became moribund and died because of the extent of tumor growth. Some fibroblast strains, such as LF1, produced smaller tumors in the kidney but even such small tumors invaded the kidney parenchyma (Figure 1). By the criteria of extensive local growth, invasiveness, and metastatic potential, it is clear that the combination of SV40 LT and Ras^G12V, without other genetic modifications, is sufficient to convert normal human fibroblasts into cancer cells.

hTERT and SV40 ST Are Required for Tumorigenic Growth in Cells Transplanted Subcutaneously

This conclusion is in disagreement with the consensus that has emerged from experiments over the past 20 years that the combination of SV40 LT and Ras^G12V is nontumorigenic in normal human fibroblasts. These data include experiments in which genes were introduced into the cells by transfection as well as retroviral transduction [1,15]. However, all of the reported studies of tumorigenicity of these cells in immunodeficient animals used subcutaneous or intramuscular injection to test malignant potential. A detailed survey of the literature on the transformation of human fibroblasts by a variety of oncogenes does not reveal any in which tumorigenicity was tested by insertion of the cells into the kidney or other internal organs.

Considering the data from the literature together with the results of the experiments reported here, we hypothesized that hTERT and SV40 ST may be required for growth of neoplastically transformed human fibroblasts under the skin but not in internal organs. To test this, we constructed a retrovirus that contains the SV40 early region (SV40 ER) in its native form. Cells transduced with this retrovirus express both SV40 LT and SV40 ST (Figure 2). To introduce hTERT, we infected cells with pBabe-hTERT followed by puromycin selection. Cells transduced with pBabe-hTERT showed strong telomerase activity, whereas cells transduced with only SV40 ER and Ras^G12V were telomerase-negative (see below).

Four different populations of cells expressing combinations of SV40 LT, SV40 ST, Ras^G12V, and hTERT were injected under the dorsal skin of immunodeficient mice. Only SV40 ER/Ras/hTERT cells formed tumors, whereas SV40 LT/Ras, SV40 ER/Ras, and SV40 LT/Ras/hTERT did not (Table 2). In the case of the latter three cell populations, a small fluorescent bleb was visible under the skin immediately following the injection. These blebs remained visible for a few days but did not enlarge and eventually could no longer be detected by fluorescence. In contrast, SV40 ER/Ras/hTERT cells produced continuously enlarging tumors that invaded into subcutaneous muscle and fat (Figure 2).

In view of the widespread use of anchorage-independent growth as a surrogate assay for tumorigenic potential, we also examined the ability of various transduced cell populations to form colonies by growth in soft agarose. Nontransduced cells did not form colonies. In agreement with observations on early-passage normal human fibroblasts transduced with oncogenic Ras and/or hTERT [21], cells transduced with SV40 LT and Ras^G12V formed colonies of
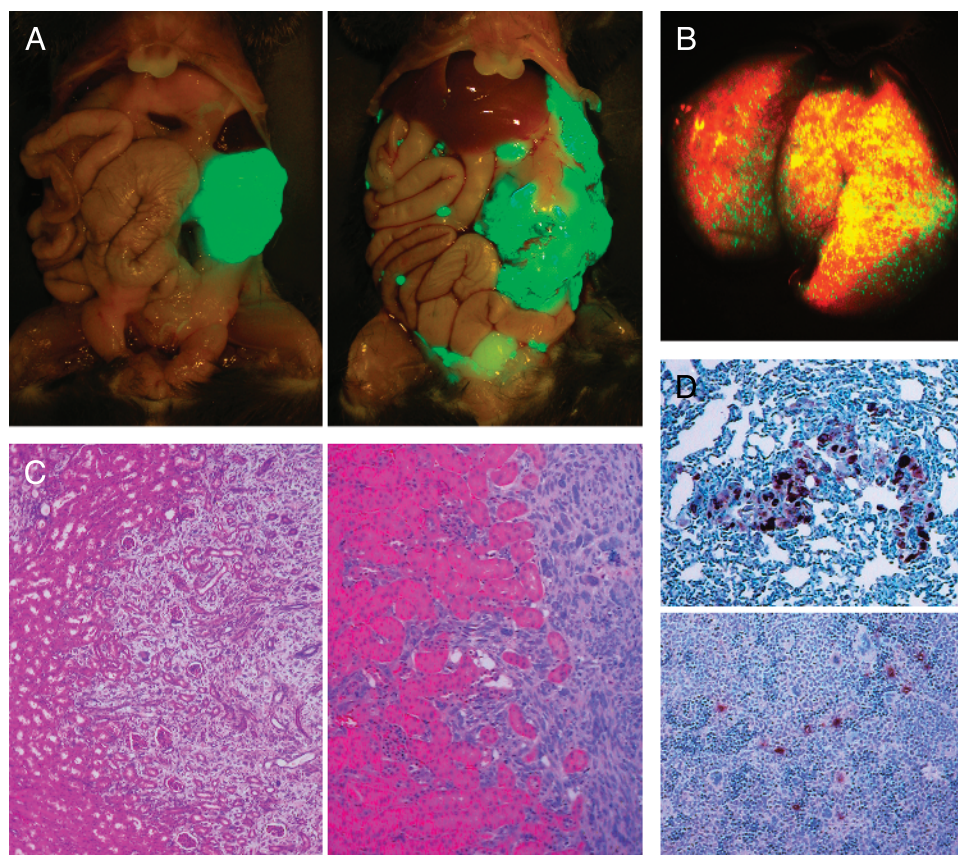
Figure 1. *Invasive and metastatic behavior of primary human fibroblasts expressing SV40 LT and Ras^{G12V}. (A) Two examples of tumors growing in the kidneys of immunodeficient mice, in one case (right) showing additional separate tumor masses within the peritoneal cavity. Photographs were obtained using blue light illumination for detection of GFP together with low-level white light for visualization of the host organs. (B) Lung metastases visualized by fluorescence. (C) Hematoxylin and eosin–stained sections of two tumors showing invasion into the kidney; on the left is a section of a large tumor formed from CRL-2703 cells and on the right is a section of a small tumor formed from LF1 cells. (D) Tumor cells in the lung (above) and spleen (below) revealed by immunocytochemistry using an antibody against SV40 LT.*

various sizes; SV40 ER/Ras/hTERT cells formed larger colonies (Figure 2). Although we did not investigate the phenomenon in great detail, we noted that those SV40 LT/ Ras–transduced fibroblast strains that produced smaller tumors in the kidney, notably LF1, also formed only very small colonies by growth in soft agarose.

Tumors Formed from SV40 LT/Ras Cells Progressively Lose Their Malignant Properties and Enter Crisis

We noted that tumors from animals sacrificed at later time points were often not larger than those sacrificed at earlier times, suggesting that tumors may stop growing or reach a stage beyond which they grow very little. To test whether tumors were losing the ability to continue to grow in immunodeficient mice, we performed the following serial transplantation experiment. We excised 1-mm fragments from primary tumors formed from SV40 LT/Ras^{G12V}–transduced HCA2 cells. Fragments were reimplanted beneath the kidney capsule of secondary host animals and also beneath the skin. Six of seven primary tumors were able to regrow to some extent in the kidneys of secondary host animals, although none of 10 was able to regrow under the skin. Secondary tumors in the kidney were much slower-growing than the

primary tumors (Figure 3). No fragments from secondary tumors could be regrown when implanted into the kidneys of tertiary host animals; only scar tissue was observed at the site of implantation. We conclude that tumors are not capable of indefinite growth and reach a stage at which they either

Table 1. Malignant Transformation of Primary Human Fibroblasts by SV40 LT and Ras.

Fibroblast Strain	PDL	Tumor Incidence	Size at 45 days (cm)
HCA2 (MJ-90)	19	10/10	0.5–2.0
HCA3 (BJ)	8	6/6	0.5–1.5
CRL-2088	11	3/3	0.3–0.5
CRL-2091	18	3/3	0.5–1.5
CRL-2094	12	3/3	0.3–0.5
CRL-2097	11	3/3	0.3–0.8
CRL-2429	21	3/3	0.5–1.0
CRL-2703	8	3/3	1.5–2.0
LF1	20	3/3	0.2–0.3

The table lists the primary human fibroblast strains used, the population doubling level (PDL) at which the cells were transduced with SV40 LT and Ras^{G12V}, the incidence of tumors when cells were implanted in the kidney, and the thickness of the tumors at the time of sacrifice of the animals.
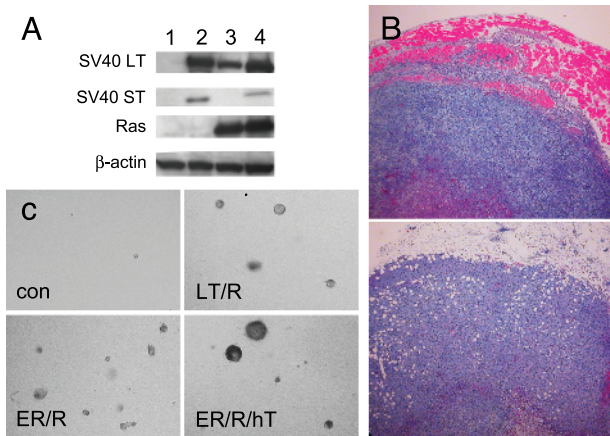
Figure 2. *Requirement for hTERT and SV40 ST for tumorigenicity when cells are injected subcutaneously in immunodeficient mice. HCA2 human fibroblasts were transduced with retroviruses encoding either SV40 LT or SV40 ER, together with Ras^{G12V} and/or hTERT. (A) Western blot analysis showing the expression of SV40 LT and SV40 ST in cells transduced with the SV40 ER retrovirus. (1) Nontransduced HCA2 cells; (2) GM847 cells (SV40-transformed cell line, used as positive control); (3) HCA2 cells transduced with SV40 LT and Ras^{G12V}; (4) HCA2 cells transduced with SV40 ER and Ras^{G12V}. (B) Two examples of subcutaneous tumors formed from HCA2 cells transduced with SV40 ER, Ras^{G12V}, and hTERT, showing invasion into muscle (above) and fat (below). (C) Colony growth in soft agarose of control and genetically modified HCA2 cells. con, control HCA2 cells; LT, SV40 LT; ER, SV40 ER; R, Ras; hT, hTERT.*

stop growing or regress. This is also accompanied by loss of malignant behavior. Primary tumors extensively invaded the kidney and some formed metastases (Figure 1). Secondary tumors showed much less invasion into the kidney (Figure 3) and we found no metastases in these animals.

We hypothesized that the limited growth potential of SV40 LT/Ras tumors was caused by replicative senescence/crisis of the tumor cells. Histologic observations of tumors formed from SV40 LT/Ras cells showed a great heterogeneity of cell size, many cells with enlarged and distorted nuclei, and many cells with abnormal mitotic chromosomes (Figure 3). Senescing human fibroblasts in culture accumulate DNA damage foci that may be visualized as colocalization of 53BP1 and γ-H2AX [22–24]. We observed large numbers of these DNA damage foci in many sections of tumors from SV40 LT/Ras^{G12V}–transduced fibroblasts (Figure 3). Tumors were dissociated into isolated cells and the tumor cells were separated from host mouse cells by flow sorting. When these cells were placed in culture, they exhibited abnormal nuclear shapes and micronucleation, anaphase bridges, and chromatin strings between interphase nuclei (Figure 3). We conclude that cells in tumors of SV40 LT/Ras^{G12V}–transduced cells enter crisis. Such tumors often had large areas of necrotic cell death, but we detected rather few cells with typical signs of apoptosis (not shown).

Restoration of Tumorigenicity by Transduction with hTERT

The progressive loss of growth and malignant properties of tumors formed from SV40 LT/Ras^{G12V}–transduced cells appeared to result from crisis in the tumor cells. We hypothesized that this was caused by lack of a telomere main-

tenance mechanism and that introduction of telomerase into the cells would maintain telomeres and thereby restore tumorigenicity. To test this, cells were isolated from primary tumors formed from SV40 LT/Ras^{G12V}–transduced HCA2 cells and were purified by cell sorting. In culture, these cells grew poorly and exhibited signs of crisis, as shown in Figure 3. They were telomerase-negative both before transplantation and after isolation from the tumor; thus, the combination of SV40 LT and Ras does not confer detectable telomerase activity (Figure 4).

Some of the telomerase-negative SV40 LT/Ras^{G12V} HCA2 cells were transduced with a retrovirus encoding hTERT. Following drug selection, the cell growth rate increased, until the cell population showed a proliferative rate at least as great as that of primary cells. These cells had a high level of telomerase activity (Figure 4). They were tumorigenic when transplanted in the kidney. The TRF length decreased progressively in HCA2 cells as they were transduced with SV40 LT and Ras^{G12V}, then transplanted to form a tumor, from which cells were isolated by flow sorting. It then increased greatly when these cells were transduced with hTERT and retransplanted to form a tumor (Figure 4). Tumors formed from hTERT-transduced cells grew rapidly and were invasive and metastatic (Figure 4). The malignant properties of the tumors became even more prominent when fragments of such tumors were serially transplanted to subsequent hosts. Tumors could be retransplanted sequentially at least three times without any evidence of loss of growth rate. Thus, hTERT rescues cells from crisis and restores full tumorigenicity.

Discussion

The human diploid fibroblast has long been popular in studies of neoplastic transformation because of its ease of isolation from human subjects and its central role in understanding replicative senescence and crisis [25,26]. Attempts to convert normal human fibroblasts into cancer cells by direct genetic modification failed until Hahn et al. [1] reported that four genes—*SV40 LT*, *SV40 ST*, *Ras*, and *hTERT*—were required for this transformation. In particular, in that study and in previous reports [15], the combination of SV40 LT and Ras was nontumorigenic by the conventional criterion of formation of tumors following subcutaneous injection of the cells in immunodeficient mice. Further experiments

Table 2. Subcutaneous Tumor Formation by Genetically Modified Primary Human Fibroblasts.

Genetic Modifications	Tumor Incidence	
	Subcutaneous	Subrenal Capsule
SV40 LT, Ras	0/12	10/10
SV40 LT, SV40 ST, Ras	0/8	4/4
SV40 LT, Ras, hTERT	0/6	nd
SV40 LT, SV40 ST, Ras, hTERT	6/6	nd

The table shows tumor formation by HCA2 cells expressing the indicated combinations of genes, when assayed by subcutaneous injection and subrenal capsule transplantation.
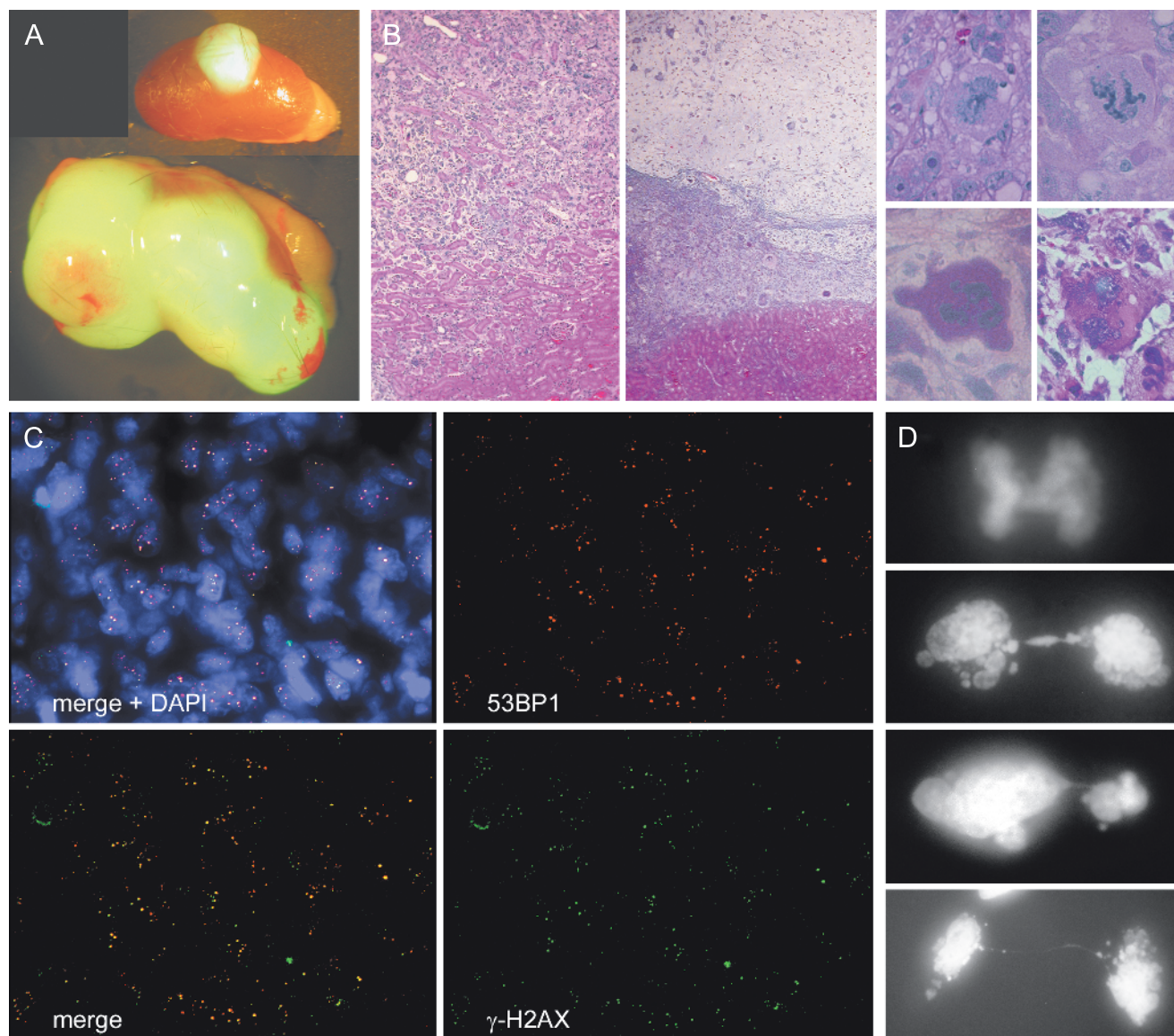nd, not determined.

Figure 3. *Loss of malignant properties in SV40 LT/Ras^{G12V} tumors associated with occurrence of crisis. (A) Comparison of primary and secondary tumors. SV40 LT/Ras^{G12V}–transduced HCA2 cells were injected under the kidney capsule and formed the lower tumor after 38 days. A 1-mm fragment of this tumor was retransplanted into a second animal and formed the upper tumor after 42 days. The two tumors are shown at the same magnification. (B) Hematoxylin and eosin–stained sections of the primary SV40 LT/Ras tumor (left) and the secondary tumor (center) together with examples of abnormal nuclei and abnormal mitotic chromosomes in secondary tumors (right). (C) DNA damage foci visualized by colocalization of 53BP1 and γ-H2AX. A primary SV40 LT/Ras tumor was fixed and sectioned as described in the Materials and Methods section, and sections were incubated with antibodies against 53BP1 (visualized with Texas Red) and γ-H2AX (visualized with fluorescein). Sections were also stained with DAPI to show nuclei. (D) Examples of an anaphase bridge and chromatin strings between interphase nuclei in tumor cells placed in culture (DAPI stain).*

in the same model—human fibroblasts and SV40 LT/SV40 ST/Ras/hTERT—have confirmed and amplified the conclusion that these is a minimal set of genes required for neoplastic transformation [27–33].

However, we demonstrate here that this conclusion is not valid when early-passage primary human fibroblasts are transduced with SV40 LT and Ras and implanted beneath the kidney capsule rather than under the skin of immunodeficient mice. Early-passage human fibroblasts transduced with SV40 LT and Ras^{G12V} produced tumors that were invasive and sometimes metastatic. Thus, using experimental conditions that are as close to the in vivo situation as

possible—the use of early-passage cells and formation of the tumor within an internal organ—both *hTERT* and *SV40 ST* are eliminated from the minimal set of genes required to convert human fibroblasts into cancer cells.

The present study used nine different strains of primary human fibroblasts—all from normal donors and all used at the earliest PD available. In agreement with previous data on early-passage fibroblasts [21], we found that Ras had a positive effect on growth; in particular, we found that Ras-transduced cells exhibited anchorage-independent growth. Later-passage cells show a radically different response to Ras—in those cells, Ras causes senescence [34]. This has

been puzzling, given the central role of oncogenic Ras in increasing proliferation, invasion, angiogenesis, and other features of tumors in vivo [2,3]. Ras-induced senescence requires the activity of p16^{INK4A} [31,35–38]. The passage-dependent induction of senescence by Ras^{G12V} is correlated with an increase in p16 as a function of PDL [21,31]. This increase in p16 appears to be a response to oxidative or other forms of damage incurred during growth in culture [39]. These results question whether p16-dependent Ras-induced senescence represents a barrier that must be overcome for cells to respond to the protumorigenic effects of Ras in a living tissue. It is important to define sets of genes that are required for neoplastic transformation using early-passage cells, thereby avoiding artificial barriers to transformation that have been acquired by the cells during growth in culture.

Evidently, the microenvironment in the kidney is ideal for the survival, growth, and vascularization of both normal and neoplastic cells. The critical features of this microenvironment have not been definitively identified; important features may be the proximity to abundant capillaries within the kidney and the positive interstitial fluid pressure [40]. This site is also ideal for revealing invasive behavior of tumorigenic cells, as malignant cells grow between renal tubules, eventually destroying the entire organ. We confirmed earlier observations that normal human fibroblasts, as well as fibroblasts expressing only SV40 LT or only Ras, have no malignant properties when transplanted in the kidney. They form a thin layer on top of the kidney parenchyma. A clear boundary with the kidney is maintained and there is no sign of invasion (data not shown). A relevant question is whether the kidney is an "ectopic" site for the growth of neoplastic fibroblasts

and that the subcutaneous site might be more "physiological." However, it should be noted that fibroblasts occur in all organs of the body and could potentially (although rarely) form a tumor in any organ. Conversely, subcutaneous injection actually places the cells beneath the panniculus carnosus and not within the dermis; they are therefore not actually within the skin.

The present experiments lead to the conclusion that both hTERT and SV40 ST are dispensable for tumorigenic growth of human fibroblasts. However, both genes are required for tumorigenic growth of cells beneath the skin, and hTERT is also required for long-term tumorigenicity of SV40 LT/Ras–transduced cells. The latter effect of hTERT is specific for the rescue of cells that have reached crisis. Whereas SV40 LT/Ras–transduced cells are clearly malignant, as evidenced by invasive and metastatic growth, which may result in the death of the animal, they are nevertheless not immortal. Growth of the tumors eventually ceases because of telomere shortening, which leads to crisis. Crisis was demonstrated by nuclear DNA damage foci and mitotic catastrophe, and by more specific indications of telomere dysfunction such as anaphase bridges and internuclear chromatin strings. Cells were rescued from this state by expression of hTERT, which conferred telomerase activity and caused telomere lengthening. Under these circumstances, hTERT restores the malignant potential of the cells and permits serial transplantation of the tumor through multiple host animals. The incidence of DNA damage foci in tumors was also greatly reduced (data not shown).

Thus, immortalization, as a feature of cancer cells, should be distinguished from other features of malignant cells. In
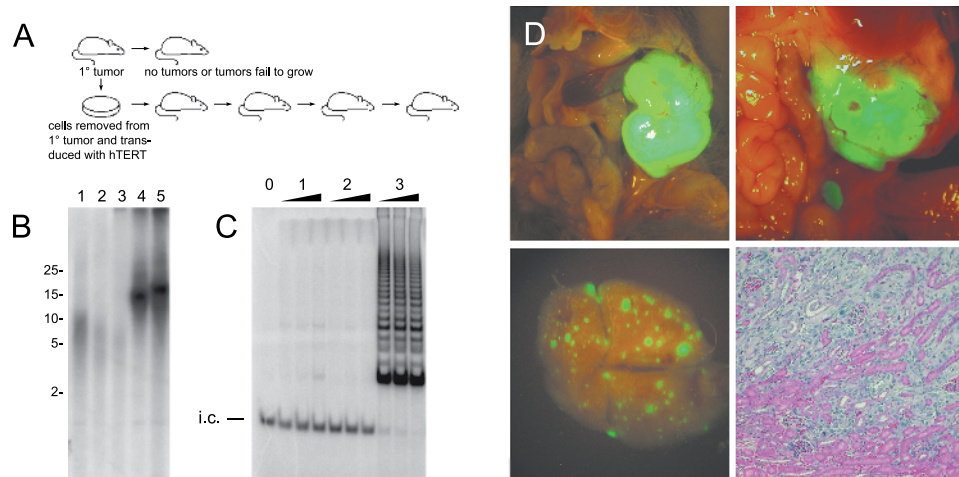

Figure 4. *Restoration of tumorigenicity by transduction with hTERT. (A) Scheme of experiments: Cells isolated from a primary SV40 LT/Ras tumor are infected with a retrovirus encoding hTERT; following drug selection, cells are retransplanted into a host animal. The tumor that forms may then be serially transplanted. (B) Southern blot of restriction enzyme-digested DNA hybridized with a telomere sequence probe. (1) HCA2 cells; (2) SV40 LT/Ras^{G12V}–transduced HCA2 cells; (3) these cells were transplanted and formed a tumor, from which cells were isolated and purified by flow sorting; (4) the tumor cells were transduced with hTERT, retransplanted to form a tumor, from which cells were isolated and flow-sorted; (5) this tumor was transplanted to another animal and regrew; cells were again isolated and flow-sorted. (C) Telomerase activity. (0) No sample; (1) HCA2 cells transduced with SV40 LT and Ras^{G12V}; (2) these cells were transplanted and formed a tumor, from which cells were isolated and purified by flow sorting; (3) these tumor cells were transduced with hTERT (50, 100, and 200 ng of protein for each sample). (D) Tumor formation from SV40 LT/Ras^{G12V}–transduced HCA2 cells following isolation of the cells from a primary tumor, transduction with hTERT, and regrowth in the subrenal capsule assay. Tumor at 38 days (upper left); tumor formed by retransplantation of a 1-mm fragment from this tumor in another animal at 28 days (upper right); lung metastases in the same animal (lower left); invasion into the kidney of a serially transplanted tumor (lower right).*

order for cells to form a lethal cancer, they must have acquired the ability to bypass normal controls on cell proliferation, the ability to invade and destroy organs, and the ability to form distant metastases. Although other properties of cancer cells are commonly observed, such as resistance to apoptosis and immortality, there is no *a priori* reason for those properties to be essential for cells to form a lethal cancer. In the SV40 LT/SV40 ST/Ras/hTERT model of neoplastic transformation of human fibroblasts, the absolute requirement for hTERT is problematic. If immortalization is the specific property conferred by hTERT, then one would expect that cells with relatively long telomeres should still be converted to tumor cells by SV40 LT/SV40 ST/Ras and that such cells would develop a requirement for telomerase only when telomeres have shortened to a critical extent, as shown here in the subrenal capsule assay. In contrast, in the subcutaneous assay, the SV40 LT/SV40 ST/Ras combination produced no tumors in extensive trials [1,29,41–43].

These data suggest that, in the subcutaneous site, hTERT has an essential role in tumorigenesis by conferring properties on cells, other than immortalization. The requirement for hTERT may reflect one or more of the many other reported effects of high-level hTERT expression in cells, including stimulation of cell proliferation and antiapoptotic effects [14]. Further studies are needed to define the cellular and molecular processes/pathways that must be targeted to permit the survival and growth of cells in the subcutaneous site, which are not needed in the subrenal capsule site. They could include resistance to stresses, such as osmotic stress, lack of nutrients, oxidative stress, and hypoxia. Because these genetic modifications are not required for tumorigenicity *per se*, genes that have been identified in the past as oncogenes (or classified as nononcogenic) based on subcutaneous injection experiments need to be restudied using growth of cells within an internal organ.

In summary, the minimal set of genetic changes required for tumorigenicity of human fibroblasts was assessed by implanting cells in immunodeficient host animals at a site that permits optimal cell survival, using early-passage cells that have not acquired artefactual barriers to neoplastic transformation. The cellular pathways targeted by SV40 large T antigen (p53 and pRb) and those targeted by activated Ras represent a minimal set of genetic alterations required for the conversion of normal human fibroblasts into cancer cells.

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